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Evolution in interacting species alters predator life history traits, behavior and morphology in experimental microbial communities

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Abstract

Predator-prey interactions heavily influence the dynamics of many ecosystems. An increasing body of evidence suggests that rapid evolution and co-evolution can alter these interactions, with important ecological implications, by acting on traits determining fitness, including reproduction, anti-predatory defense and foraging efficiency. However, most studies to date have focused only on evolution in the prey species, and the predator traits in (co-)evolving systems remain poorly understood. Here we investigated changes in predator traits after ~600 generations in a predator-prey (ciliate-bacteria) evolutionary experiment. Predators independently evolved on seven different prey species, allowing generalization of the predator's evolutionary response. We used highly resolved automated image analysis to quantify changes in predator life history, morphology and behavior. Consistent with previous studies, we found that prey evolution impaired growth of the predator, although the effect depended on the prey species. In contrast, predator evolution did not cause a clear increase in predator growth when feeding on ancestral prey. However, predator evolution affected morphology and behavior, increasing size, speed and directionality of movement, which have all been linked to higher prey search efficiency. These results show that in (co-)evolving systems, predator adaptation can occur in traits relevant to foraging efficiency without translating into an increased ability of the predator to grow on the ancestral prey type.

Keywords: predator-prey interactions, trait evolution, ciliate physiology, microbial model systems, experimental evolution

Introduction

Predator-prey interactions are ubiquitous across ecosystems. Predation has been widely studied at an ecological level [1-3], and recent research also shows that this interaction can be strongly altered by rapid evolution of anti-predatory defense in the prey [4] as well as by counter-adaptations in the predator [5-7], even though selection may be asymmetric resulting in slower evolutionary change for the predator [8]. Moreover, owing to population growth-defense tradeoffs, rapid evolution of the prey and adaptation to predation can result in frequency-dependent selection of defended and undefended prey types as a function of predator population size [9-11], an example of eco-evolutionary feedback dynamics. Common to this spectrum of evolutionary, co-evolutionary and eco-evolutionary dynamics is that these dynamics are all driven by natural selection acting on fitness-relevant traits.

Predation can be described by three main phases, namely prey search, capture and ingestion [12]. These three phases are shaped by key traits in predator-prey systems, including those influencing offence and defense level, and all these traits can be subject to evolutionary change [13]. Offense level is determined by sensory faculties and speed enabling location and capture of prey, and defense level by the capacity for predator avoidance and escape prior to ingestion as well as physicochemical obstruction of ingestion and digestion [12]. Adaptations in defense and offense, in turn, combined with associated tradeoffs, modulate the reproduction (i.e. life history traits) of both parties [14]. Examples abound of the study of the different phases of predation, and adaptation in both predator and prey life history traits. For example, the timing and population dynamics of many insectivorous bird species are tightly coupled to the dynamics of their prey insect species [15]. Olive baboon sleeping site choice and behavior (sharing sleeping sites between multiple baboon groups) in Kenya were recently linked to decreased contact and capture rate by leopards [16]. Co-evolution has been hypothesized to occur between Northern Pacific rattlesnakes and California ground squirrels whereby

68 venom resistance in squirrels is matched by increased venom effectiveness in rattlesnakes based on
69 field data supportive of local adaptation of the traits [17].
70
71 The empirical examples of evolving predator-prey interactions described above cannot be used to
72 experimentally investigate (co-)evolution in predator-prey systems due to the long generation times
73 of the species. In contrast, microbial systems offer a unique opportunity to study predator-prey
74 dynamics, as they include efficient (high prey capture rate) predators and allow for high replication
75 as well as experimental approaches capturing both ecological and evolutionary dynamics. Microbial
76 predator-prey systems show many key characteristics found also in other predator-prey systems, such
77 as offense by speed [18] and defense by avoidance of detection [19], escape [20], or physicochemical
78 obstruction of ingestion or digestion (for an overview, see [12]). Defense level has also been
79 demonstrated to evolve in controlled setups [21, 22]. However, to our knowledge, there exist little to
80 no empirical studies examining offense mechanisms subject to rapid evolution in microbial predator-
81 prey systems.
82
83 Here we employed an experimental evolution approach to test the influence of ~600 generations of
84 predator-prey interaction on predator traits, using a microbial (ciliate-bacteria) model system. Since
85 predator-prey dynamics are characterized by the intrinsically linked dynamics of both interaction
86 partners, we inspected the influence of both prey and predator evolution on predator traits. To find
87 general patterns in predator traits independently of any specific prey species, as most predators have
88 multiple prey species [23], we used seven different prey species that were all separately evolved with
89 the predator. We expected rapid evolution of anti-predatory defense in the prey to cause impairment
90 of predator growth [7, 14]. We expected predator evolution to be weaker in line with the life-dinner
91 principle [8, 24] positing that the prey experiences stronger selection pressure since its survival (life)
92 directly depends on defense while the predator can afford a certain measure of unsuccessful prey

93 encounters (dinner postponement). Asymmetric selection can result in dynamics other than classic
94 arms race dynamics such as frequency-dependent cycling of traits [5], which have also been observed
95 in microbial predator-prey systems [22]. Nevertheless, instead of escalation where predators alone
96 impose selection pressure, we expected to also observe predator evolution, since co-evolution has
97 been demonstrated to occur in bacteria-ciliate systems, in line with the Red Queen hypothesis [7, 14,
98 25].
99

100 Material and methods

101 We studied the evolutionary dynamics of one focal predator species (the ciliate *Tetrahymena*
102 *thermophila*) and seven of its bacterial prey species in all seven combinations of predator-prey species
103 communities, as well as dynamics in prey-species populations only. We ran predator-prey
104 evolutionary experiments over about 600 predator generations, and assessed evolutionary effects on
105 life history, morphology and behavior using common garden experiments.
106

107 Strains and culture conditions

108 The seven prey species used in this study are listed in Table 1. In addition to four taxa previously
109 used as models in predator-prey studies, three strains were chosen based on representing genera
110 associated with ciliate predators in natural habitats or potentially exhibiting different anti-predatory
111 defense mechanisms (Table 1). Since all the strains represent unique genera, they are referred to by
112 their genus name in the text.
113

114 We used a single strain of the asexually reproducing ciliate *Tetrahymena thermophila* 1630/1U
115 (CCAP) [26] as a generalist predator capable of consuming all the prey species. *Tetrahymena*
116 *thermophila* is a ciliate species characterized by a facultative sexual reproductive cycle and nuclear
117 dualism, where the cells contain a small diploid non-expressed germline nucleus (micronucleus) and

a larger highly polyploid somatic nucleus (macronucleus), derived from the micronucleus after sexual reproduction [27]. Only the macronuclear DNA is the only expressed and hence determines the phenotypic characteristics of *Tetrahymena* cells [27]. The micronucleus is only relevant for sexual reproduction. The species can be either maintained under settings of recurrent sexual reproduction, or as asexual lineages only. The *Tetrahymena* strain used in our experiment had been maintained in serial propagation for many years before the experiments. Sexual reproduction only occurs when induced by starvation [28], and because this was not the case during its long-term maintenance, the strain only underwent asexual reproduction. During asexual reproduction micronuclei and macronuclei divide independently from each other [27]. It has been noted that, when cultured for a long time asexually, the micronuclei can degrade [29] and have subsequent negative effects on the genotype's fitness during a possible sexual reproduction, or even lead to genotypes losing their ability to sexually reproduce. However, given that micronuclei are never expressed and only play a role in sexual reproduction [27], and also given that we do not induce or study the genotype's ability to reproduce sexually, this possible degradation of the micronucleus does not have consequences on fitness as measured in our setting. We also note that it is a common practice to use *Tetrahymena* cell lines with non-functional micronuclei, as described in the standard handbook for *Tetrahymena* cell biology work [29]. In all of these cases, the serial propagation is not problematic as long as one is not inducing sexual reproduction. Hence, any evolution observed on the predator level in this experiment stems either from mutations or selection on existing variation in the macronuclear DNA. Furthermore, as the macronucleus is highly polyploid (n = 45), and chromosomes divide randomly during asexual reproduction [27], cells are relatively buffered to the effects of single maladaptive mutations, and can undergo relatively rapid purging of maladaptive mutations or selection for increased copies of adaptive mutations. This together with the absence of sexual reproduction, which can be affected by serial propagation [29], makes it highly unlikely that the serial propagation setup in the experiment would itself strongly influence the evolutionary dynamics of the predator.

Table 1. Bacterial strains used in this study.

Strain*	Rationale for species selection
Escherichia coli ATCC 11303	model prey [30]
Janthinobacterium lividum HAMBI 1919	pre-/post-ingestion defense: toxin release [12]
Sphingomonas capsulata HAMBI 103	model prey [31]
Brevundimonas diminuta HAMBI 18	realistic habitat [32]
Pseudomonas fluorescens SBW25 [33]	model prey [34]
Comamonas testosteroni HAMBI 403	pre-ingestion defense: oversize [12]
Serratia marcescens ATCC 13880	model prey [31]

*ATCC = American Type Culture Collection; HAMBI = HAMBI mBRC = Microbial Domain Biological Resource Centre HAMBI, University of Helsinki, Finland

Prior to the experiments, all bacterial stocks were kept at –80 °C and ciliate stocks were cultured axenically in proteose peptone yeast extract (PPY) medium containing 20 g of proteose peptone and 2.5 g of yeast extract in 1 L of deionized water. During the evolutionary experiment, cultures were kept at 28 °C (± 0.1 °C) with shaking at 50 r.p.m.

Predator-prey evolutionary experiment

The evolutionary experiment was started using a small aliquot (20 μL) of a 48-h bacterial culture started from a single colony and 10,000 ciliate cells (approx. 1,700 cells mL^{-1}) from an axenic culture. Each bacterial strain was cultured alone and together with the ciliate predator (three replicates each, with the exception of six replicates for *Comamonas*) in batch cultures of 20 mL glass vials containing 6 mL of 5 % KB medium, with 1 % weekly transfer to fresh medium.

Every four transfers (28 days), bacterial and predator densities were estimated using optical density (1 mL sample at 600 nm wavelength) as a proxy for bacterial biomass and direct ciliate counts (5 × 0.5 μL droplets using light microscopy) as used in this context and described previously [34-36], and samples were freeze-stored with glycerol at –20 °C for later analysis. Since predators do not survive freeze-storage in these conditions, at time points 52 and 89 weeks, predator cultures were made axenic

by transferring 400 μL into 100 mL of PPY medium containing an antibiotic cocktail (42, 50, 50 and 33 μg mL^{-1} of kanamycin, rifampicin, streptomycin and tetracycline, respectively) and stored in liquid nitrogen. Axenicity was controlled for by plating on agar plates containing 50 % PPY medium where all experimental bacterial strains grow. The liquid nitrogen storage protocol was modified from a previously used protocol [29] and included starving a dense ciliate culture in 10 mM Tris-Hcl solution (pH 7) for 2–3 days, centrifugation (1700 g, 8 min, 4 ºC), resuspension of the pellet in 1 mL of leftover supernatant, and the addition of 4 mL of sterile 10 % DMSO. The resultant solution was transferred to cryotubes in 0.3 mL lots, and frozen in a –20 °C freezer at a rate of –1 °C/minute using a Mr. Frosty™ Freezing Container (Thermo Scientific) for cell preservation before transferring to liquid nitrogen.

Sample collection and preparation

We isolated the populations for the current experiment at time point 89 weeks (approx. 20 months). With the minimal assumption that populations multiply by 100-fold (dilution rate) until reaching the stationary phase, each weekly transfer interval represents 6.64 generations for both prey and predator [37], constituting a total minimum of ~600 generations. Community dynamics are shown in Supporting Figures S1 and S2 and show clear differences in population size between different prey species.

Bacteria were restored from freeze-storage by transferring 20 μL into 5 mL of 5 % KB medium and culturing for 72 h. Predators were restored from liquid nitrogen by thawing cryotubes in a 42 °C water bath for 15 s, followed by the addition of 1 mL of 42 °C PPY medium. The cryotube contents were then transferred to a petri dish containing PPY medium at room temperature. Upon reaching a high density (approx. 48 h), predators were transferred to 100 mL of PPY medium and cultured to a high density (approx. seven days). To ensure that the antibiotic treatment or the liquid nitrogen storage and

revival procedures do not contribute to potential differences between the ancestral predator and evolved predator lines, the axenic ancestral predator was subjected to identical procedures and was revived at the same time as the evolved lines. These culturing steps representing over 10 generations should remove the influence of non-genetic changes in predator traits caused by phenotypic plasticity [38].

Physiological measurements

To test bacterial and ciliate performance and traits, we used a combination of automated video analysis, optical density measurements and flow cytometry. To separate evolutionary responses on the predator and prey level, we tested performance of both evolved and ancestral bacteria with evolved and ancestral ciliates for all evolved lines reciprocally. To do so, we prepared 12 50 mL falcon® tubes by adding 20 mL of 5 % KB medium. Three of these were inoculated with ancestral bacteria and ancestral ciliates, three with ancestral bacteria and evolved ciliates, three with evolved bacteria and ancestral ciliates and the remaining three with evolved bacteria and evolved ciliates. We placed the falcon® tubes in a 28 °C incubator, rotating on a shaker at 120 r.p.m. After inoculation, the samples were left to grow for a period of 12 days, to allow populations to grow to equilibrium density. Over the course of these 12 days, we took a total of 10 samples from each culture for analyzing population density dynamics of bacteria and ciliates, and morphological and behavioral metrics for the ciliates. We sampled cultures by gently shaking the culture, to ensure it was well mixed, and subsequently pipetting out 200 μl from the mixed culture.

Bacterial density measurements

Bacterial density was determined through measurement of both optical density and through flow cytometry. Flow cytometric analyses, were based on established protocols [39, 40] which facilitate distinction between living bacterial cells and background signals (e.g. dead cells or abiotic matter).

For flow cytometry, we sampled 50 μL of all cultures, diluted 1:1000 using filtered Evian water and transferred 180 μL of the diluted samples to a 96-well-plate. We then added 20 μL of SybrGreen to strain the cells and measured bacterial cell counts using a BD Accuri™ C6 flow cytometer. As the inner diameter of the needle from the flow cytometer was 20 μm, and hence smaller than typical ciliate cell sizes, it is highly unlikely that ciliate cells were accidentally measured during flow cytometry. Also, given that bacterial densities were typically between one to five orders of magnitude larger than ciliate densities, even an occasional measurement of ciliate cells would have a negligible effect on bacterial density estimates. The full protocol can be found in the Supporting Information. For optical density measurement, we sampled 50 μL of all cultures, diluted 1:10 using filtered Evian water, and measured absorbance at 600 nm using a SpectroMax 190 plate reader.

Ciliate density and trait measurements

For measuring ciliate density, we performed video analysis [41] using the BEMOVI R-package [42]. We followed a previously established method [43] where we took a 20 s video (25 fps, 500 frames) of a standardized volume using a Leica M165FC stereomicroscope with circular lighting and mounted Hamamatsu Orca Flash 4.0 camera. We then analyzed the videos using BEMOVI [42, 44], which returns information on the cell density, morphological traits (longest and shortest cell axis length) and movement metrics (gross speed and net speed of cells, as well as turning angle distribution). The video analysis script, including used parameter values, can be found in the Supporting Information.

Data analysis

All statistical analyses were done using the R statistical software (version 3.5.1) [45]. To obtain the reported *F*- and *p*-values for predator traits, we performed ANOVA for the best linear models constructed for the different traits as described below.

Predator trait space

To visualize whether the full set of trait data displayed structure depending on the evolutionary history of the predator and prey species, t-distributed stochastic neighbor embedding (t-SNE) was performed for each prey species separately using the Rtsne package [46] with a perplexity parameter of 3 owing to small sample size.

Beverton-Holt model fit

For analyzing the population growth dynamics of the ciliates, we implemented the Beverton-Holt population growth model [47] (Figure S3) using a Bayesian framework in Rstan [48], following methods used by [49, 50] . This function has the form of:

$$\frac{dN}{dt} = \left(\frac{r_0 + d}{1 + \alpha N} - d\right) N$$

With r_0 being the intrinsic rate of increase, α the intraspecific competitive ability and d being the death rate in the population. Model code for fitting this function can be found on a Github repository (doi: 10.5281/zenodo.2658131). For fitting this model, we needed to provide prior information for r_0, d and equilibrium density K. The intraspecific competitive ability α was later derived from the other parameter values as:

$$\alpha = \frac{r_0}{Kd}$$

The priors (lognormal distribution) of the model were chosen in such a way that mean estimates lay close to the overall observed means, but were broad enough so the model was not constrained too strongly.

- Equilibrium population density K: $ln(K) \sim normal(9.21, 0.5)$

- Intrinsic rate of increase r_0: $ln(r_0) \sim normal(-2.3, 0.5)$
- Rate of mortality d: $ln(d) \sim normal(-2.3, 0.5)$

Models were run with a warmup of 2,000 iterations and a chain length of 8,000 iterations.

Life history trait analysis

We analyzed the estimates of the life history traits obtained from the Beverton-Holt model fit (r_0, α, and K) using linear models and model selection. We first constructed a full model with life history traits being a function of bacterial evolutionary history (evolved/ancestor), ciliate evolutionary history (evolved/ancestor) and bacterial species (seven species factors) in a full interaction model. Next, we used automated bidirectional model selection using the step function (stats package version 3.5.1) to find the best model. To avoid bias due to starting point, we fit the model both starting from the intercept model and the full model, and if model selection resulted in different models, we used AICc comparison (MuMIn R-package, version 1.42.1 [51]) to select the model with the smallest AICc value.

Morphological and behavioral trait analysis

Morphological and behavioral data was available for every time point during the growth curve, and since we know these traits can be plastically strongly affected by density [52, 53], we had to take density into account in the model. We hence separated the analysis into two steps: first, we identified key points in the growth curves (early phase, mid-log phase and equilibrium density phase) and analysed the traits for these particular points. Secondly, we fit models over all data, but taking bacterial (using flow cytometry data) and ciliate densities into account as covariates in the statistical analysis.

We defined the early phase as the second time point in the time series, equilibrium density phase as the first time point where density was larger than 99 % of *K*, or alternatively the highest density, and the mid-log phase as the point between the early and equilibrium density phase where density was closest to 50 % of *K*. We then created statistical models for the traits (major cell axis size, gross speed of cells and turning angle distribution) as a function of bacterial evolutionary history (evolved/ancestor), ciliate evolutionary history (evolved/ancestor) and bacterial species (seven species factors) including a full interaction for the data at the particular time point. Next, we used automated bidirectional model selection to find the best fitting model. This was done separately for all three phases (early, mid-log and equilibrium density phase). We again performed model selection both starting from the intercept model and full model, and compared the 2 models using AICc comparison to identify the best model.

We then created models using all the data, where we fit major cell axis size, gross speed and turning angle distribution as a function of bacterial evolutionary history (evolved/ancestor), ciliate evolutionary history (evolved/ancestor) and bacterial species (seven species factors), ciliate population density (ln-transformed, continuous) and bacterial population density (ln-transformed, continuous), including a full interaction. For turning angle, we also did a log10 transformation of the turning angle distributions, as fitting the model on untransformed data leads to a strong deviation on the qqplot. Next, we used automated bidirectional model selection using the step function starting from intercept model and full model, and compared the 2 models using AICc comparison to select the best model.

Results

The t-SNE maps (Figure 1) showed that the evolutionary history of the predator and prey species frequently resulted in predator divergence in trait space. Importantly, this divergence evolved from a

single ancestral predator population, which was subjected to co-culture with different prey species. The full results for all statistical analyses presented below to assess this divergence in detail are available in the Supporting Information.

Prey evolution drove changes in the life history traits of the predator, including intrinsic rate of increase (r_0), equilibrium density (K) and competitive ability (α), although the presence and strength of the effect depended on the bacterial species (ANOVA, r_0: prey evolution $F_{1,78} = 15.32$, $p < 0.001$; prey evolution × prey species $F_{6,78} = 9.03$, $p < 0.001$; K: prey evolution × prey species $F_{6,80} = 13.7$, $p < 0.001$; α: prey evolution $F_{1,78} = 4.79$, $p = 0.031$, prey evolution × prey species $F_{6,78} = 5.40$, $p < 0.001$; Tables S1–S3 and S7–S9; Figure 2). The intrinsic rate of increase of ciliates (r_0) was generally lower in presence of evolved bacterial prey compared to ancestral prey, with the notable exception of *Serratia*, where intrinsic rate of increase was higher in presence of evolved prey (Table 2; Figure 2). For three species (*Janthinobacterium*, *Brevundimonas* and *Pseudomonas*), evolved predators had a higher intrinsic rate of increase (r_0) on evolved prey compared to ancestral prey (Figure 2). Changes in population equilibrium density (K) were highly dependent on species, with four species (*Janthinobacterium*, *Brevundimonas*, *Comamonas* and *Serratia*) showing higher population equilibrium density in presence of evolved prey compared to ancestral prey, and the remaining three (*Escherichia*, *Sphingomonas* and *Pseudomonas*) showing decreased population equilibrium density in presence of evolved prey compared to ancestral prey. Competitive ability (α) typically decreased in presence of evolved prey compared to ancestral prey, with the exception of *Pseudomonas*, where competitive ability was higher in presence of evolved bacteria compared to ancestral bacteria. Notably, for *Escherichia*, *Janthinobacterium* and *Serratia*, the competitive ability (α) of evolved predators was higher in the presence of evolved prey compared to ancestral prey (Figure 2).

Table 2. Predicted change in intrinsic rate of growth (r_0), population equilibrium density (K) and competitive ability (α) in presence of evolved bacteria compared to ancestral bacteria according to the linear models. The r_0-, K-, and α-ratios are calculated as the predicted trait value (r_0, K, or α) in presence of evolved bacteria divided by the predicted trait value in presence of ancestral bacteria. Note that for the K-ratio, since predator evolution is excluded during model selection, predictions for ancestral and evolved predators are identical.

Prey species	**Predator evolution**	**r_0-ratio**	**K-ratio**	**α-ratio**
E. coli	Ancestor	0.788	0.885	0.881
E. coli	Evolved	0.943	0.885	1.08
J. lividum	Ancestor	0.912	1.06	0.849
J. lividum	Evolved	1.09	1.06	1.04
S. capsulata	Ancestor	0.381	0.517	0.730
S. capsulata	Evolved	0.457	0.517	0.893
B. diminuta	Ancestor	0.974	1.18	0.815
B. diminuta	Evolved	1.17	1.18	0.997
P. fluorescens	Ancestor	0.904	0.835	1.07
P. fluorescens	Evolved	1.08	0.835	1.31
C. testosteroni	Ancestor	0.475	1.26	0.374
C. testosteroni	Evolved	0.569	1.26	0.457
S. marcescens	Ancestor	1.09	1.16	0.930
S. marcescens	Evolved	1.31	1.16	1.14

In contrast to life history traits, which were affected by prey evolution alone, morphological and behavioral traits of the predator were affected by predator evolution (Figure 3). However, the effect size of predator evolution was also strongly dependent on predator density (for the movement metrics gross speed and turning angles) or both predator and prey density (for the biovolume metric cell size). Evolved predators were slightly but significantly larger than ancestral predators (ANOVA: predator evolution $F_{1,767} = 7.87$, $p = 0.005$). Although there was a significant effect indicating that this was modulated by the evolutionary history of the prey (ANOVA: prey evolution $F_{1,767} = 4.85$, $p = 0.033$), the associated effect size was much smaller than predator evolution. On average, evolved predators were 39.12 μm larger than ancestral predators, and predators where on average 1.629 μm smaller in presence of evolved prey compared to ancestral prey. The effect of predator evolution also depended strongly on prey densities (ANOVA: log prey density × predator evolution $F_{1,767} = 6.87$, $p = 0.009$;

358 Figure 3). The strongest differences in cell size between ancestral and evolved predators were
359 observed at low prey densities (cell sizes 1.2–1.3 times larger for evolved compared to ancestral
360 ciliates) whereas the effects were negligible at high prey densities (approximately equal size for
361 evolved and ancestral ciliates; Tables S4 and S10; Figures 3 and S4–S6).
362
363 The gross movement speed of predators depended on the interplay between predator density and
364 predator or prey evolutionary history. Evolved predators had, on average, up to 1.25 times higher
365 speed compared to ancestral predators. However, this effect occurred for evolved predators at high
366 predator densities, whereas at low predator densities, movement speed was approximately similar for
367 ancestral and evolved ciliates (ANOVA: predator density $F_{1,763} = 116.20, p < 0.001$; predator evolution
368 $F_{1,763} = 1.90, p = 0.239$; predator evolution × predator density $F_{1,763} = 4.36, p = 0.037$; Figure 3). This
369 effect was partially counteracted by prey evolution by driving speed to a lower rate at increasing
370 predator densities (ANOVA: prey evolution $F_{1,763} = 2.17, p = 0.141$; prey evolution × predator density
371 $F_{1,763} = 5.46, p = 0.020$). The movement speed of ciliate cells was also dependent on identity of the
372 prey species, with ciliates moving slower when subjected to three particular prey species
373 (*Janthinobacterium*, *Pseudomonas* and *Serratia*; ANOVA: prey evolution $F_{6,763} = 9.11, p < 0.001$;
374 Tables S5 and S11; Figure S7). Finally, predator evolution altered cell turning angle distribution
375 across prey species such that evolved predator lines moved in straighter trajectories (ANOVA:
376 predator evolution $F_{1,56} = 10.15, p = 0.001$). This effect was again highly dependent on predator
377 population size, with evolved predators turning approximately 0.92 times the turning rate of ancestral
378 predators at low predator density, but turning equally as much at high predator density (ANOVA:
379 predator density $F_{1,763} = 33.90, p < 0.001$; predator evolution × predator density $F_{1,763} = 5.44, p = 0.02$;
380 Figure 3). The effect of predator population size was also dependent on prey species, such that for
381 three prey species (*Janthinobacterium*, *Pseudomonas* and *Serratia*), evolved predators moved even

straighter (less turning) at higher predator densities (ANOVA: predator density × prey species $F_{1,763}$ = 6.76, $p < 0.001$; Tables S6 and S12; Figures S8–S10).

Discussion

We quantified the contribution of predator and prey evolution to predator trait change across seven different prey species in a 20-month (~600 predator generations) co-culture experiment. Prey evolution frequently led to changes in predator life history traits, decreasing intrinsic growth rate, equilibrium density or competitive ability, while not affecting morphological or behavioral traits in the predator. Interestingly, the strength of the effect and the life history trait affected depended on the prey species. These results may be influenced by different growth dynamics, defense levels or defense mechanisms of the different prey species (Table 1, Figures S1 and S2) [12].

For two of the predator life history traits, intrinsic rate of increase (r_0) and competitive ability (α),the trait was impaired with evolved compared to ancestral prey in all expect for two cases (*Serratia* for r_0 and *Pseudomonas* for α). This could be caused by any mechanism of prey defense evolution decreasing effective prey population size or increasing prey handling time, including cell aggregation of bacterial prey frequently shown under ciliate predation, [54, 55]. While a similar result was also observed for population equilibrium density (K) with three prey species (*Escherichia*, *Sphingomonas* and *Pseudomonas*), intriguingly, the remaining four prey species (*Brevundimonas*, *Comamonas*, *Janthinobacterium* and *Serratia*) showed higher K in the presence of evolved compared to ancestral prey. This counterintuitive result may be caused by resource use evolution, which can occur rapidly in bacterial evolutionary experiments [37] but differ in magnitude between bacterial (i.e. prey) species. In this situation, a sufficient increase in prey population size could sustain a higher predator population size despite anti-predatory defense evolution.

Consistent with the Red Queen hypothesis, evolved predators displayed both behavioral and morphological changes linked to prey foraging efficiency. Increased swimming speed and body size were observed for evolved predators with certain prey species, and predators evolved to swim in straighter trajectories across the different prey species. Increased swimming speed and decreased cell turning (i.e. moving in straighter trajectories) have both been linked to prey search efficiency [18, 56, 57], and in line with this, ciliates have been shown to display decreased cell turning and increased speed at low food concentrations [58]. The role of increased body size is less clear but may also be related to increased prey search efficiency since swimming speed can be a function of body size [18, 56]. All these evolutionary trait changes in the predator are consistent with being adaptations to decreased food availability owing to anti-predatory defense evolution in the prey species.

Interestingly, against our expectation based on the Red Queen hypothesis, we did not find detectable levels of adaptation in predator life history traits when prey-evolved predators fed on their respective ancestral prey species. This could be indicative of asymmetry of selection [5, 22] such that predators experience weaker selection pressure compared to prey owing to the life-dinner principle [8] whereby prey species rely on adaptation (needed to stay alive) more strongly than predators (needed to increase energy uptake). Asymmetric evolutionary change for ciliate predators could also result from smaller population size (in the order of 10^4 mL^{-1} for ciliates compared with 10^8 mL^{-1} for bacteria), larger genome size (>100 Mb for *T. thermophila* compared to <10 Mb for bacteria) or more complex genomic architecture limiting adaptive mutation supply compared to the bacterial prey [59].

There are two ways asymmetric selection could account for our unexpected result regarding the lack of evolution in ciliate life-history traits. First, the offense-related traits (morphology and behavior) where predator evolution was observed may simply not have improved sufficiently to be detectable as increased predator growth on ancestral prey using our methods. Although the culture conditions

were mostly identical between the serial passage experiment and ciliate physiology measurements (same culture medium, temperature, covering 7-day time span representing serial passage culture cycle), it is also possible that minor differences in experimental conditions (different culture vials, volumes and shaking parameters) or the revival of ciliates from liquid nitrogen storage could have introduced noise in the data masking ciliate evolution in life history traits. Second, rapid evolution in the prey species may have changed basic features of the prey population early on in the experiment, such as causing cell aggregation widely documented to evolve rapidly in similar setups [21, 22, 54, 55]. An improved ability of the predator to feed on defended prey with altered characteristics may not allow for an improved ability to also feed on ancestral prey. For instance, higher speed and directionality of movement may be useful when feeding on unevenly distributed prey aggregates while not causing a benefit when feeding on prey as homogeneously distributed single cells (food being always closely available). Alternatively, as a more complex explanation, a steepening growth-offense trade-off during co-evolution [14] could cause stunted growth in co-evolved high-offense-level predators, which may therefore only display a net fitness improvement against prey in a recent evolutionary state. Since our sample material represents a snapshot from the end-point of a long-term (co-)evolutionary experiment, further experiments would be needed to assess the dynamics of predator trait change over time to test these hypotheses.

Our findings have implications for interpreting data from (co-)evolving predator-prey systems. First, the pronounced impairment of predator growth traits upon prey evolution together with the lack of clear improvements in the ability of evolved predators to feed on ancestral prey types support the asymmetric selection hypothesis. Second, the occurrence of predator evolution in other key traits for predator-prey interaction despite this suggests that tracking ecological changes alone may result in an underestimation of predator evolution [60, 61]. A deeper understanding of predator-prey evolutionary

456 dynamics is therefore likely to critically depend on the identification and examination of key traits
457 for the interaction, preferably over time and including both interaction partners.
458

Data Availability

All code and pre-processed data needed to reproduce the ecological and evolutionary analyses are available via Dryad (10.5061/dryad.08kprr4zr).

Author Contributions

Designed co-evolutionary experiment: J.C. and T.H. Performed and managed experiment: J.C. Designed physiological measurements: F.M., E.A.F., and F.A. Performed physiological measurements: F.M., E.A.F., and F.A. Analyzed data: F.M. and J.C. Wrote manuscript draft: J.C. All authors interpreted results and participated in improving the manuscript.

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Conflict of Interest Statement

We declare we have no competing interests.

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623 **Figure legends**

624

625 **Figure 1.** t-SNE map of contribution of predator and prey evolutionary history to predator
626 divergence in trait space. The traits included in the analysis encompass life history (intrinsic growth
627 rate, equilibrium density and competitive ability), morphology (cell size and biovolume) and
628 behavior (speed and cell turning angle distribution).

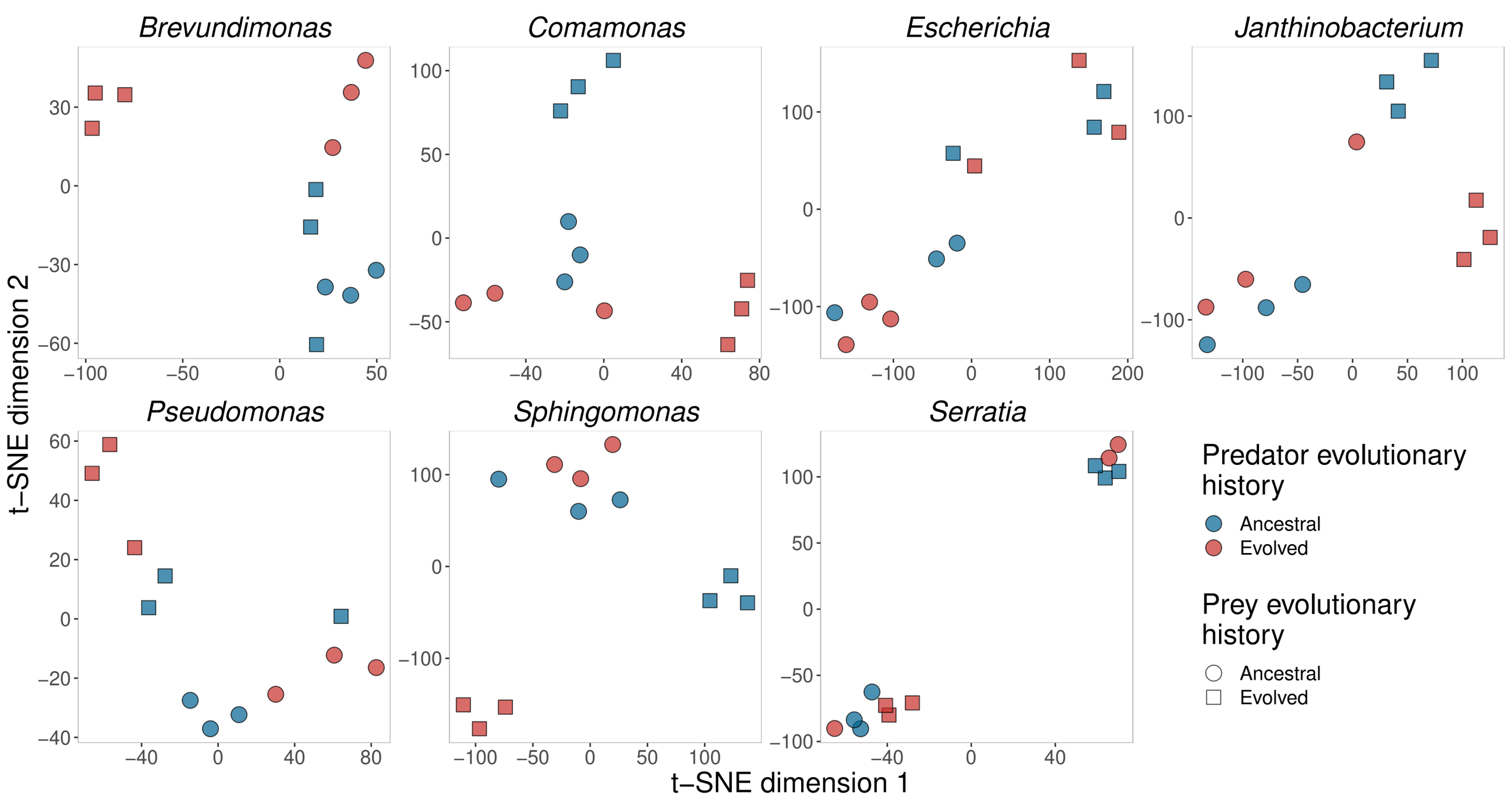
629

630 **Figure 2.** Reaction norms showing effect of evolving predator-prey interaction on life-history traits
631 of predator (data points with linear model estimate ± 95 % confidence intervals.; N = 3 except 6 for
632 *Comamonas*). The life-history traits for predators are parameters of Beverton-Holt continuous-time
633 population models fitted to data, and include intrinsic growth rate (r_0), equilibrium density (K) and
634 competitive ability (α). The reaction norms for predators (one strain of the ciliate *Tetrahymena*
635 *thermophila*) feeding on ancestral or evolved prey (seven bacterial strains indicated by genus name)
636 are depicted separately for ancestral and evolved predators (color coding). Predators evolved with a
637 particular prey taxon have always been coupled with ancestral or evolved populations of the same
638 taxon, while the ancestral predator is the same for all prey taxa.

639

640 **Figure 3.** Ratios of the predicted trait values of the linear models (cell size, gross cell speed and
641 turning angles) for the evolved predator divided by the ancestral predator at different prey densities
642 (5 %, 50 % and 95 % quantiles) and predator densities (5 %, 50 % and 95 % quantiles). Ratios
643 represent how ciliate traits differ between evolved and ancestral ciliates, with values of one
644 meaning evolved and ancestral ciliates are identical, values larger than one meaning higher trait
645 values for evolved strains, and values smaller than one higher trait values for ancestral ciliates.

646


Brevundimonas
Comamonas
Escherichia
Janthinobacterium
Pseudomonas
Sphingomonas
Serratia
t–SNE dimension 2
t–SNE dimension 1
Predator evolutionary history
Ancestral
Evolved
Prey evolutionary history
Ancestral
Evolved

Brevundimonas *Comamonas* *Escherichia* *Janthinobacterium* *Pseudomonas* *Serratia* *Sphingomonas*

Life history traits

Intrinsic rate of increase, $\log r_0 \left(\frac{1}{h}\right)$

−1.5
−2.0
−2.5
−3.0
−3.5

Population equilibrium density, $\log K \left(\frac{\text{individuals}}{\text{mL}}\right)$

9.6
9.2
8.8
8.4

Competitive ability, $\log \alpha \left(\frac{\text{mL}}{\text{h} \cdot \text{individuals}}\right)$

−10.5
−11.0
−11.5
−12.0
−12.5

Ancestral Evolved

Evolutionary history of prey

Evolutionary history of predator

Ancestral
Evolved

5 % prey density
50 % prey density
95 % prey density
Cell size
Gross cell speed
Turning angle
1.2
1.1
1.0
0.9
Trait ratio ($\frac{\text{Evolved predator}}{\text{Ancestral predator}}$)
5 %
50 %
95 %
Predator density (quantile)
Evolutionary history of prey
Ancestral
Evolved